

# Expression of CHI3L1 and CHIT1 in osteoarthritic rat cartilage model. A morphological study

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## Abstract

Osteoarthritis is a degenerative joint disease, which affects millions of people around the world. It occurs when the protective cartilage at the end of bones wears over time, leading to loss of flexibility of the joint, pain and stiffness. The cause of osteoarthritis is unknown, but its development is associated with different factors, such as metabolic, genetic, mechanical and inflammatory ones. In recent years the biological role of chitinases has been studied in relation to different inflammatory diseases and more in particular the elevated levels of human cartilage glycoprotein 39 (CHI3L1) and chitotriosidase (CHIT1) have been reported in a variety of diseases including chronic inflammation and degenerative disorders. The aim of this study was to investigate, by immunohistochemistry, the distribution of CHI3L1 and CHIT1 in osteoarthritic and normal rat articular cartilage, to discover their potential role in the development of this disease. The hypothesis was that the expression of chitinases could increase in OA disease. Immunohistochemical analysis showed that CHI3L1 and CHIT1 staining was very strong in osteoarthritic cartilage, especially in the superficial areas of the cartilage most exposed to mechanical load, while it was weak or absent in normal cartilage. These findings suggest that these two chitinases could be functionally associated with the development of osteoarthritis and could be used as markers, so in the future they could have a role in the daily clinical practice to stage the severity of the disease. However, the longer-term *in vivo* and *in vitro* studies are needed to understand the exact mechanism of these molecules, their receptors and activities on cartilage tissue.

## Introduction

Osteoarthritis (OA) also known as degenerative arthritis or degenerative joint disease or osteoarthrosis is the most common form of arthritis, affecting millions of people around the world. It affects about 8 million people in the United Kingdom and nearly 27 million people in the United States.<sup>1</sup> Often called wear-and-tear arthritis, OA occurs when the protective cartilage at the ends of bones wears down over time.<sup>2</sup> Although OA can damage any joint in the body, the disorder most commonly affects joints in the hands, neck, lower back, knees and hips. OA is a group of mechanical abnormalities involving degradation of joints including not only the articular cartilage but also the subchondral bone.<sup>3</sup> OA gradually worsens with time, and no cure exists. Treatments can slow the progression of the disease, relieve pain and improve joint function.<sup>4</sup> Symptoms of OA include loss of flexibility, limited movement, pain and swelling within the joint.<sup>5</sup> The condition results from injury to the cartilage, which normally absorbs stress and covers the bones, so they can move smoothly. A variety of causes, hereditary, developmental, metabolic, and mechanical deficits, may initiate processes leading to loss of cartilage. When bone surfaces become less protected by cartilage, bone may be exposed and damaged. As a result of decreased movement secondary to pain, regional muscles may atrophy, and ligaments may become more lax. The main symptom is pain, causing loss of ability and often stiffness. Pain is generally described as a sharp ache, or a burning sensation in the associate muscles and tendons. Treatment generally involves a combination of exercise, lifestyle modification, and analgesics;<sup>5</sup> if pain becomes debilitating, joint replacement surgery may be used to improve the quality of life.<sup>6</sup> For most people, the cause of OA is unknown, but metabolic, genetic, chemical, inflammation and mechanical factors play a role in its development.<sup>7</sup> For this reason, in the present study, we investigated, in an *in vivo* osteoarthritic rat model, the human cartilage glycoprotein 39 (GP-39, YKL-40), also known as CHI3L1 and the chitotriosidase (CHIT1). Mammalian chitinases belong to the glycohydrolase family 18, which have evolved to hydrolyze chitin, a polymer of N-acetylglucosamine.<sup>8,9</sup> The family of chitinases includes members both with and without glycohydrolase enzymatic activity against chitin. CHIT1 is a true chitinase possessing chitinolytic (glycohydrolase) activities.<sup>10</sup> In contrast, chitinase-like lectins (Chi-lectins) or chitinase-like proteins (C/CLPs), including chitinase 3-like-1 (CHI3L1, YKL40, HC-gp39), show enzymatic activity despite the retention and conservation of the substrate-binding cleft of the chitinases.<sup>11</sup> For

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the majority of the mammalian chitinases important biological roles in chronic inflammatory diseases have been identified.<sup>12-14</sup> So far, CHIT1 is the best-characterized true chitinase from a clinical and biological perspective. Elevated levels have been reported in a variety of diseases including infections, chronic inflammation and degenerative disorders.<sup>15,16</sup> The sources of secreted CHIT1 are abnormal lipid-laden macrophages formed in tissues of patients with Gaucher's disease.<sup>17</sup> This molecule correlated strongly with disease symptoms and is used to monitor the efficacy of therapy.<sup>18</sup> Recently, it was hypothesized that cellular alteration in Gaucher's disease produced a proinflammatory milieu leading to bone destruction through enhancement of monocyte differentiation to osteoclasts and improvement of osteoclasts resorption activity.<sup>19</sup> To confirm

this data it was demonstrated that the chitinases, CHIT1 and CHI3L1, are closely related to the process of osteoclastogenesis and the digestion of bone matrix via MMP9.<sup>20</sup> Despite various theories having been proposed to explain the disruption of bone homeostatic balance in Gaucher's disease, implying dysfunction of osteoclasts, osteoblasts and mesenchymal cells,<sup>21-23</sup> to date the effect of CHIT1 remains nearly unexplored. Only one study has shown that, in periprosthetic soft tissue from patients with osteolysis the expression of alternative macrophage activation markers (CHIT1, CCL18) was increased in comparison to OA controls.<sup>24</sup> Interestingly this finding indicated that the activation of alternative macrophage is involved in osteolysis and suggested a correlation between CHIT1 and osteolytic lesions.<sup>22</sup> In contrast to CHIT1, some evidence reports increased levels of CHI3L1 protein and/or mRNA in patients with a wide spectrum of pathologies.<sup>25</sup> The CHI3L1 is a glycoprotein secreted by articular chondrocytes, synoviocytes and macrophages. Serum and synovial fluid CHI3L1 levels are elevated in inflammatory diseases and correlate with the degree of joint destruction in rheumatoid arthritis. CHI3L1 is a candidate auto antigen in rheumatoid arthritis and is important in the capacity of cells to respond to and cope with changes in their environment.<sup>26</sup> Recently Einarsson and coauthors stated that chondrocytes of human osteoarthritic cartilage secrete the inflammation associated chitolectin CHI3L1.<sup>27</sup> CHI3L1, is a major secretory protein of human chondrocytes in cell culture. CHI3L1 mRNA is expressed by cartilage from patients with rheumatoid arthritis, but is not detectable in normal human cartilage.<sup>28</sup> Moreover, it was observed that in patients with myeloma elevated serum concentrations of CHI3L1 aggravated bone destruction and were associated with an increase of bone resorption activity hastening the progression of bone disease.<sup>29</sup> The aim of this study was to investigate, by immunohistochemistry the distribution of CHIT1 and CHI3L1 in osteoarthritic (n=20) and normal (n=10) rat articular cartilage, collected from femoral condyles after anterior cruciate ligament transection (ACLT), to discover a potential role for CHI3L1 and CHIT1 in osteoarthritic cartilage and to improve a new possible therapeutic concept for treating inflammatory joint diseases. The hypothesis is that the expression of chitinases could increase in OA disease.

## Materials and Methods

### Breeding and housing of animals

In our study, we used thirty 6-months-old healthy male Sprague Dawley rats (Charles River Laboratories, Milan, Italy), with an average body

weight of 200±40 g. Rats were individually housed in polycarbonate cages (cage dimensions: 10.25"W x 18.75"D x 8"H) during the entire period of the study and were housed at controlled temperature (20-23°C) and humidity, with free access to water and food and photoperiod of 12 hours light/dark. All surgical procedures for anterior cruciate ligament transection were performed in accordance with the method previously described.<sup>30,31</sup> The ACLT procedure was made under total anesthesia, 30 mg/Kg Zoletil 100 + altadol 5 mg/kg + maintenance mixture of O<sub>2</sub> and isoflurane 2-2,5%, (Vibrac, Milan, Italy). Postoperatively, the animals were permitted free cage activity without joint immobilization and treated with the administration of an antibiotic, Convenia® 0.1 mL/kg, (Vibrac). The 30 animals were distributed in two different groups: 10 rats for control group without ACLT, and 20 rats for OA group with ACLT. The control group consists of two subgroups: control normal group (5 rats) without surgical treatment and sham-operated control group (5 rats), rats that have received exactly the same surgical procedure as the exper-

imental group, without ACLT. The OA group instead was made up of 20 rats with surgical treatment submitted to ACLT to induce OA model. During the experiment the possible suffering of the animals was evaluated through the clinical appearance of the animal (fur, weight, lameness, consumption of food and water) and their possible elimination from the trial was evaluated once a day. The animals from all groups at 3 months after the surgical procedures were sacrificed by intracardial Pentothal® injection 30-40 mg/kg (Biochemie, Kundl, Austria); under Furane 2%®-narcosis (Abbott Lab., Maidenhead, UK). Both femurs were explanted, cleaned of soft tissues and the samples were used to perform histomorphometric evaluations. Cartilage tissue were used to perform histological and immunohistochemical analyses. All procedures conformed to the guidelines of the Institutional Animal Care and Use Committee of the University of Catania. The experiments were conducted in accordance with the European Community Council Directive (86/609/EEC) and the Italian Animal Protection Law (116/1992).

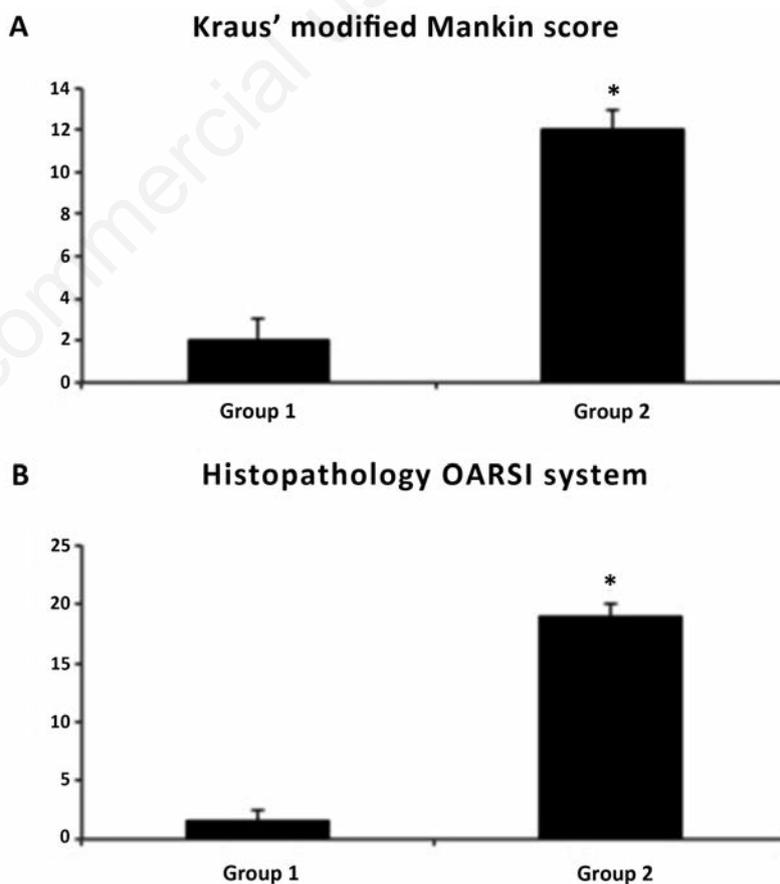


Figure 1. A) Kraus' modified Mankin score between groups; results are presented as the mean ± SEM; Student's *t*-test was used to evaluate the significance of the results; \*P<0.01, when compared to the control group. B) Histopathology OARSI system between groups; results are presented as the mean ± SEM; Student's *t*-test was used to evaluate the significance of the results; \*P<0.01, when compared to the control group.

## Histomorphometric analysis

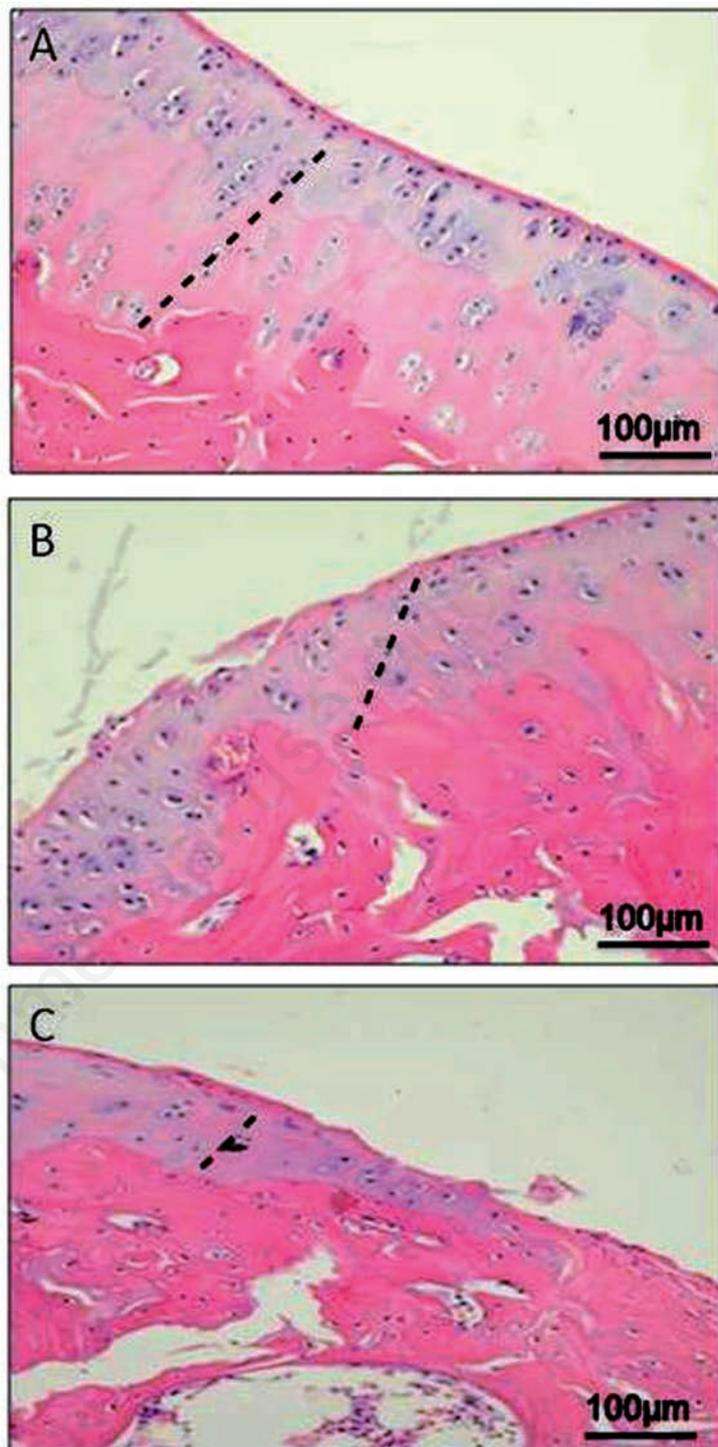
Femurs were explanted and cleaned of soft tissues as previously described.<sup>32</sup> Histomorphometric analysis was performed on the total number of rats used and specifically on both medial and lateral femoral condyles from all groups (untreated and treated animals). Histomorphometry was performed with image analysis, Kontron KS 300 software (Kontron Electronics, Eching bei Munchen, Germany). Two blinded investigators (2 anatomical morphologists) made the analyses. We assumed that the evaluations were correct if there were no statistically different values between the investigators. Fifteen fields randomly selected from each section were analyzed. The semi-quantitative histological grading criteria of Kraus' modified Mankin score<sup>33,34</sup> and histopathology OARSI system<sup>35</sup> were used.

## Histology

Samples were fixed in 10% neutral buffered-formalin (Bio-Optica, Milan, Italy), following overnight washing and routinely embedded in paraffin as previously described.<sup>36</sup> After wax infiltration the tissue samples were orientated in the cassettes in the same direction. Sections 4-5  $\mu\text{m}$  thick were cut from paraffin blocks using a rotary manual microtome (Leica RM2235, Milan, Italy) and mounted on silane-coated slides (Menzel-Gläser, Braunschweig, Germany) and stored at room temperature. Slides were dewaxed in xylene, hydrated using graded ethanol, and stained as previously described<sup>37</sup> for routine histological evaluation by hematoxylin and eosin (H&E) staining for the morphological structure. The sections were examined with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRC5, Carl Zeiss).

## Immunohistochemistry

For immunohistochemical analysis slides were processed as previously described.<sup>38</sup> Briefly, the slides were dewaxed in xylene, hydrated using graded ethanol and were incubated for 30 min in 0.3%  $\text{H}_2\text{O}_2$ /methanol to quench endogenous peroxidase activity and then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica). The sections were heated (5 min x 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica), using a microwave oven (750 W) to unmask antigenic sites. The blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA, Sigma-Aldrich, Milan, Italy) in PBS for 1 h in a moist chamber. BSA was used as a blocking agent to prevent non-specific binding of the antibody. Following blocking, the sections were incubated overnight at 4°C with goat polyclonal GP-39 antibody (CHI3L1), work dilution in PBS 1:100 (sc-30465, Santa Cruz Biotechnology, Inc.,



**Figure 2.** A) H&E staining demonstrated absence of structural alterations in control cartilage, group 1 without anterior cruciate ligament transection (ACLT); the dashed line represents the layers (thickness) of hyaline healthy cartilage; in the superficial zone, cells are flat and small; in the middle and deep zone, cells are organized in columns; the tidemark is evident. B) H&E staining demonstrated signs of structural alterations in moderate OA cartilage (group 2 with ACLT); the dashed line represents the layers (thickness) of hyaline cartilage; the structural alterations included a reduction of cartilage thickness of the superficial and the middle zones; the tidemark is almost intact. C) H&E staining demonstrated signs of structural alterations in severe OA (group 3 with ACLT); the dashed line represents the layers (thickness) of hyaline cartilage; severe OA cartilage demonstrated deep surface clefts, disappearance of cells from the superficial zone, cloning, and a lack of cells in the intermediate and deep zone, which are not arranged in columns; the tidemark is no longer intact and the subchondral bone shows fibrillation; cartilage is completely replaced by fibrocartilaginous, scar-like tissue with fibroblast like cells.

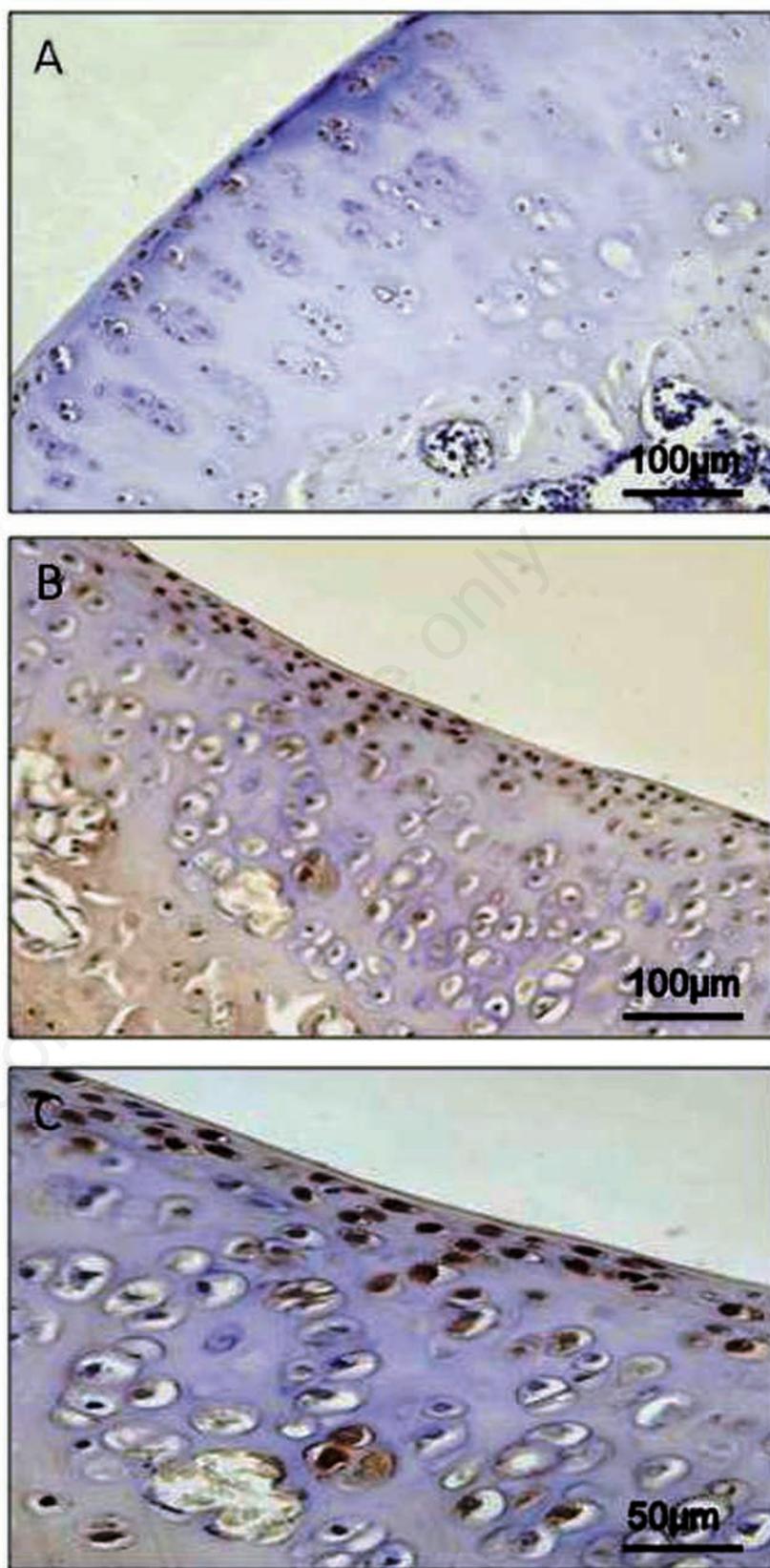
Dallas, TX, USA) and rabbit polyclonal Chitotriosidase (CHIT1) antibody, work dilution in PBS 1:100 (sc-99033, Santa Cruz Biotechnology, Inc.). Immune complexes were then treated with a biotinylated link antibody (HRP-conjugated anti-goat and anti-rabbit were used as secondary antibodies) and then detected with peroxidase labeled streptavidin, both incubated for 10 min at room temperature (LSAB+ System-HRP, K0690, Dako, Glostrup, Denmark). The immunoreaction was visualized by incubating the sections for 2 min in a 0.1% 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako). The sections were lightly counterstained with Mayer's Hematoxylin (Histolab Products AB, Goteborg, Sweden) mounted in GVA mount (Zymed Laboratories Inc., San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss).

### Evaluation of immunohistochemistry

The CHI3L1 and CHIT1-staining status was identified as either negative or positive. Immunohistochemical positive staining was defined as the detection of brown chromogen on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane and evaluated as previously described.<sup>38</sup> Stain intensity and the proportion of immunopositive cells were also assessed by light microscopy. Intensity of staining (IS) was graded on a scale of 0-4, according to the following assessment: no detectable staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3, very strong staining = 4. The percentage of antibodies immunopositive cells (Extent Score=ES) was independently evaluated by 2 investigators (2 anatomical morphologists) and scored as a percentage of the final number of 100 cells in five categories: <5% (0); 5-30% (+); 31-50% (++); 51-75% (+++), and >75% (++++). Counting was performed under Zeiss Axioplan light microscope at x200 magnification. In case of disputes concerning the interpretation, the case has been revised to reach a unanimous agreement, as previously described.<sup>39</sup> Digital pictures were photographed with a digital camera (Canon, Tokyo, Japan) at 20x, 40x and 60x magnification. Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at a protein level. Positive control for both antibodies consisted of rat liver sections. Sections treated with PBS without the primary antibodies served as negative controls. Positive immunolabeling for antibodies were nuclear/cytoplasmic.

### Computerized morphometric measurements and image analysis

Fifteen fields, randomly selected from each section, were analyzed and the percentage area



**Figure 3.** A) CHI3L1 immunohistochemistry specimen from control cartilage, group 1 without anterior cruciate ligament transection (ACLT), exhibited a weak/absent (ES=+; IS=1) immunostaining in chondrocytes from rat femoral articular cartilage. B) CHI3L1 immunohistochemistry specimen from moderate/severe OA cartilage (group 2, with ACLT) exhibited a very strong (ES=++++; IS=4) immunostaining in chondrocytes from rat femoral articular cartilage (superficial and middle zone). C) Magnification of panel B.

stained with CHI3L1 and CHIT1 antibodies was calculated using image analysis software (AxioVision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany), which quantifies the level of staining intensity of positive immunolabelling in each field. Digital micrographs were taken using the Zeiss Axioplan light microscope (Carl Zeiss) using objective lens of magnification  $\times 20$ , *i.e.*, total magnification 400) fitted with a digital camera (AxioCam MRc5, Carl Zeiss); evaluations were made by two blinded investigators, whose evaluations were assumed to be correct if values were not significantly different. In case of dispute concerning interpretation, the samples were re-evaluated in order to reach a unanimous agreement.

### Statistical analysis

Statistical analysis was performed using SPSS software (SPSS® release 16.0, IBM, Chicago, IL, USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. Comparisons between two means were tested with the Student's *t*-test, whilst comparison between more than two groups was tested using analysis of variance (ANOVA) and Bonferroni's test. P-values of less than 0.05 were considered statistically significant; p-values of less than 0.01 were considered highly statistically significant. Data are presented as the mean  $\pm$ SEM as previously described.<sup>38</sup> Cohen's kappa was applied to measure the agreement between the two observers and averaged to evaluate overall agreement as previously described.<sup>38</sup>

## Results

### Histomorphometric analyses

The histomorphometric parameters performed in group 1 (without ACLT), confirmed that the animals demonstrated no sign of cartilage degeneration with an intact and normal cartilage structure, whilst in group 2 (with ACLT) the animals demonstrated more serious pathological changes to the cartilage, OA moderate and severe, in fact horizontal cleavage tears or flaps

and deep lesions were present. Group 2 confirmed the development of articular degenerative processes, which were significantly different from the control group, as confirmed by Kraus' modified Mankin score (Figure 1A), and histopathology OARSI system (Figure 1B). The inter-observer variability among three observers for the MANKIN system showed a similar good intra-class correlation coefficient (ICC>0.82) as for the OARSI system (ICC>0.72). Repeat scoring by investigators showed very good agreement (ICC>0.96). The surface represented by lesion

depth was the parameter where all investigators showed an excellent agreement. Other parameters such as cellularity and tidemark had greater inter-reader disagreement.

### Histology

The histological (H&E staining) analysis of cartilage from group 1 (without ACLT), showed a preserved morphological structure (Figure 2A). This contrasts with group 2 (with ACLT), moderate OA cartilage where structural alterations included a reduction of cartilage thick-

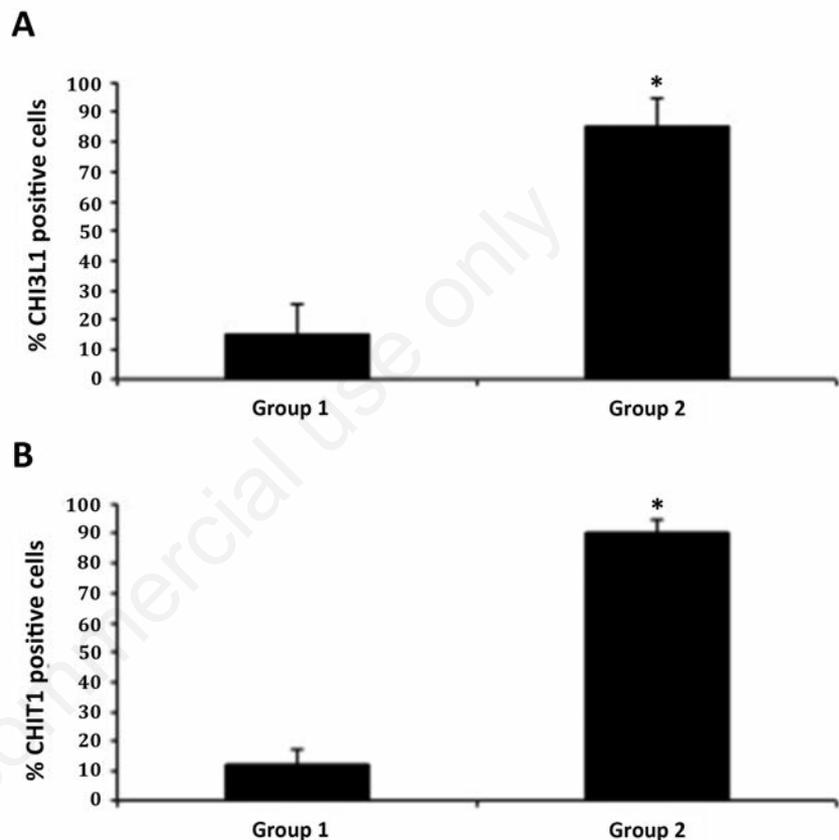


Figure 4. A) Immunohistochemistry: percentage of CHI3L1 positive cells out of the total number of cells counted in control group and in treated group; results are presented as the mean  $\pm$ SEM; Student's *t*-test, was used to evaluate the significance of the results; \*P<0.01, when compared to the control group. B) Immunohistochemistry: percentage of CHIT1 positive cells out of the total number of cells counted in control group and in treated group; results are presented as the mean  $\pm$ SEM; Student's *t*-test, was used to evaluate the significance of the results; \*P<0.01, when compared to the control group.

Table 1. Evaluation of CHI3L1 and CHIT1 immunostaining.

Groups	Intensity of CHI3L1 immunostaining and percentage of CHI3L1 immunopositive cells	Intensity of CHIT1 immunostaining and percentage of CHIT1 immunopositive cells
Control rats without ACLT	Weak/absent immunostaining (ES=+; IS=1)	Weak/absent immunostaining (ES=+; IS=1)
Experimental rats with ACLT	Very strong immunostaining (ES=++++; IS=4)	Very strong immunostaining (ES=++++; IS=4)

ACLT, Anterior cruciate ligament transection; IS, intensity of staining; ES, extent score; IS was graded on a scale of 0-4, according to the following assessment: no detectable staining (0), weak staining (1), moderate staining (2), strong staining (3), very strong staining (4). The percentage of lubricin immunopositive cells was independently evaluated by 3 investigators (2 anatomical morphologists and one histologist) and scored as a percentage of the final number of 100 cells in five categories: <5% (0); 5-30% (+); 31-50% (++); 51-75% (+++), and >75% (++++).

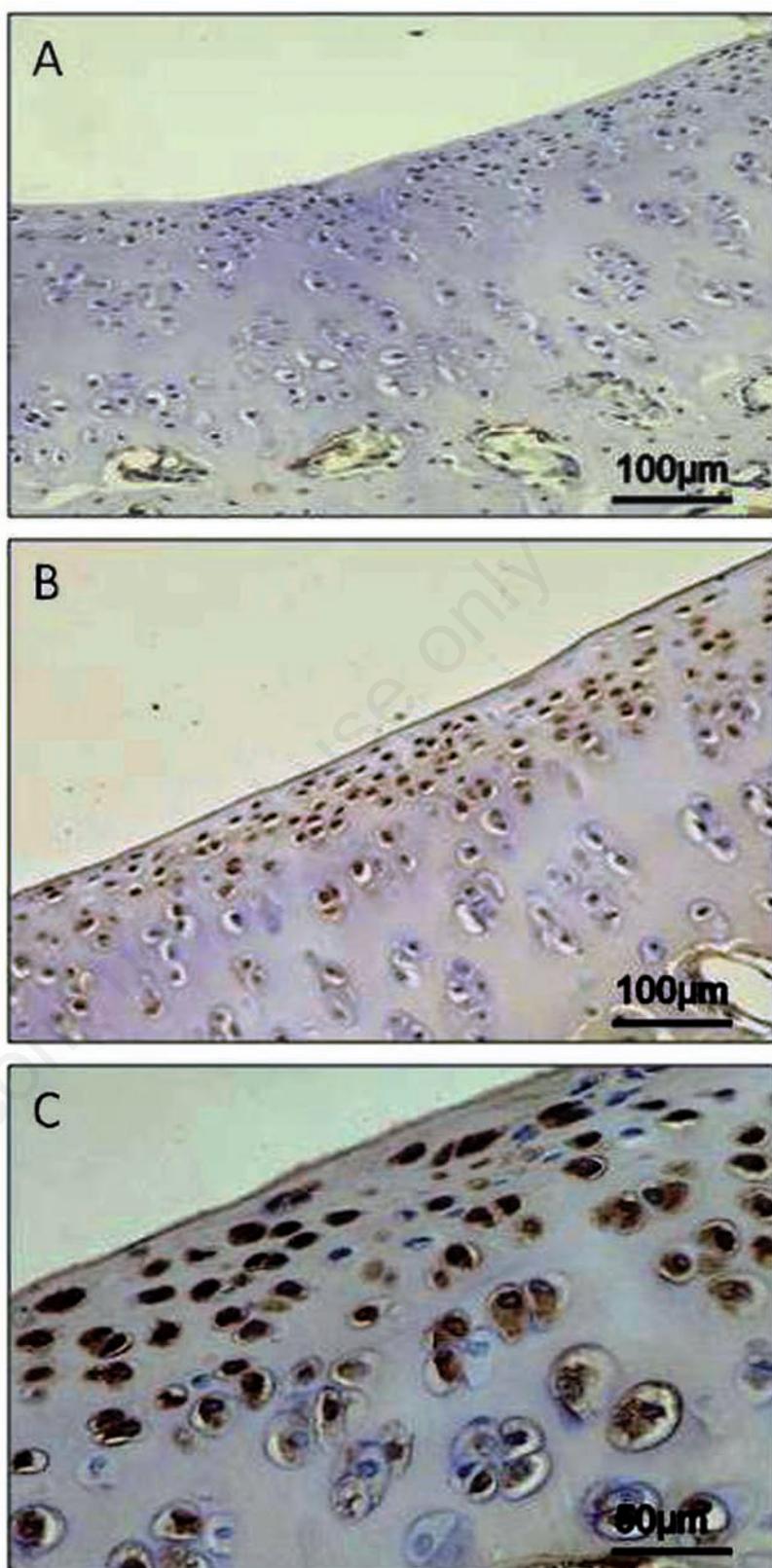
ness of the superficial and the middle zones (Figure 2B). The structure of the collagen network is damaged, which leads to reduced thickness of the cartilage. The chondrocytes are unable to maintain their repair activity with subsequent loss of the cartilage tissue. In severe OA, group 2 (with ACLT), the cartilage demonstrated deep surface clefts, disappearance of cells from the tangential zone, cloning, and a lack of cells in the intermediate and radial zone, which are not arranged in columns. The tidemark is no longer intact and the subchondral bone shows fibrillation (Figure 2C). Moreover, while the surface of healthy hyaline cartilage appears white, shiny, elastic and firm, in OA the surface becomes dull and irregular.

### Immunohistochemical observations

CHI3L1 and CHIT1 were evaluated by immunohistochemical staining in cartilage of all groups (Table 1). Different patterns of immunopositive cells in the sets of specimens were seen. This immunohistochemical staining was found in chondrocytes of osteoarthritic cartilage mainly in the superficial and middle zone of the cartilage rather than the deep zone, while it was weak/absent in normal cartilage. CHI3L1 immunolabeling was weak/absent in cartilage tissue samples from group 1, without ACLT, [(Figure 3A); (ES=+; IS=1)], and very strong in group 2, with ACLT, moderate/severe OA [(Figure 3 B,C); (ES=++++; IS=4)]. No immunostaining was observed in the negative control (ES=0; IS=0) treated with PBS without the primary antibody (*data not shown*). The percentage of CHI3L1-positive cells was observed among groups ( $P < 0.01$  vs others) (Figure 4A). Interobserver agreement, measured as Kappa coefficient, was 0.95 (almost perfect). CHIT1 immunolabeling was weak/absent in cartilage tissue samples from group 1, without ACLT, [(Figure 5A); (ES=+; IS=1)], and very strong in group 2, with ACLT, moderate/severe OA [(Figure 5 B,C); (ES=++++; IS=4)]. No immunostaining was observed in the negative control (ES=0; IS=0) treated with PBS without the primary antibody (*data not shown*). The percentage of CHIT1-positive cells observed among groups ( $P < 0.01$  vs others) (Figure 4B). Interobserver agreement, measured as Kappa coefficient, was 0.92 (almost perfect).

### Discussion

Articular cartilage injuries are one of the most challenging problems in musculoskeletal medicine due to the poor intrinsic regenerative capacity of this tissue.<sup>40</sup> OA represents a major clinical and scientific challenge for clinicians and biologists due to the limited repair capacity of articular cartilage,<sup>41</sup> rich in matrix



**Figure 5.** A) CHIT1 immunohistochemistry specimen from control cartilage, group 1 without anterior cruciate ligament transection (ACLT), exhibited a weak/absent (ES=+; IS=1) immunostaining in chondrocytes from rat femoral articular cartilage. B) CHIT1 immunohistochemistry specimen from moderate/severe OA cartilage (group 2, with ACLT) exhibited a very strong (ES=++++; IS=4) immunostaining in chondrocytes from rat femoral articular cartilage (superficial and middle zone). C) Magnification of panel B.

proteins but also avascular.<sup>42,43</sup> Articular cartilage defects are increasingly common among the elderly population and cause pain, reduced joint function and significant disability.<sup>44,45</sup> In this study, we have demonstrated that CHI3L1 and CHIT1 are expressed in osteoarthritic rat cartilage model (Figure 6). CHI3L1 has been linked to tissue remodelling,<sup>46,47</sup> joint injury,<sup>48</sup> and significantly elevated levels of CHI3L1 protein have been detected in serum and synovial fluid from OA patients.<sup>49,50</sup> The plethora of evidence showing that CHI3L1 stimulates proliferation of connective tissue cells and modulates expression levels of chemokines and metalloproteases in inflammatory fibroblasts, and that enhances chemotaxis of endothelial cells<sup>51,52</sup> strongly indicate that CHI3L1 plays a crucial role in stromal cells not only in inflammatory conditions. Additionally, *in vitro* studies demonstrated that CHI3L1 is secreted by osteosarcoma<sup>53</sup> and during osteoclast differentiation and bone digestion.<sup>54,20</sup> The findings showing a correlation between CHI3L1 expression and the development of primary and metastatic tumours further support the idea that CHI3L1 plays a role in the development and progression of a variety of malignancies.<sup>55,56</sup> Considerable experimental evidence reports the central role of CHIT1 in the expanding spectrum of disorders suggesting that overproduction of CHIT1 could exert deleterious effect in many degenerative disorders.<sup>57</sup> This concept is also sustained by our previous findings in which we observed that genetic variation within the CHIT1 gene was strongly associated with human longevity and with several phenotypes of healthy aging,<sup>58</sup> and that a functional polymorphism in the CHIT-1 gene protects from non alcoholic fatty liver disease (NAFLD) progression.<sup>59</sup> Recently, we demonstrated that CHIT1 production is not a macrophages peculiarity and that CHIT1 may play an important role in the process of bone remodeling.<sup>20</sup> These findings suggested that, patients with elevated serum levels of CHIT1 and CHI3L1 may have a more increased osteolytic activity and a faster progression of the disease. Indeed, silencing CHIT1 and CHI3L1 with siRNA resulted in a significant decrease in bone resorption activity and transfection with CHIT1 or CHI3L1 siRNA and co-transfection with both decreased the levels of the pro-differentiative marker MMP9.<sup>20</sup>

In the present study the histomorphometric parameters performed in group 1 (without ACLT), confirmed that the animals demonstrated no sign of cartilage degeneration with an intact and normal cartilage structure, whilst in group 2 (with ACLT) the animals showed more serious pathological changes to the cartilage, OA moderate and severe, in fact horizontal cleavage tears or flaps and deep lesions were present, as confirmed by Kraus' modified

Mankin score and histopathology OARS system. These results were corroborated by histological examination. Immunohistochemical analysis showed that CHI3L1 and CHIT1 staining was found in chondrocytes of osteoarthritic cartilage mainly in the superficial and middle zone of the cartilage rather than the deep zone. There was a tendency for a high number of positive chondrocytes in areas of the femoral condyles with a considerable biomechanical load. Our results are in accordance with Connor and co-authors who declared that CHI3L1 is expressed in osteophyte and diseased human osteoarthritic cartilage, but not in non-diseased one, and its distribution within the tissue changes, as disease progresses.<sup>60</sup> The number of chondrocytes with a positive staining for both CHI3L1 and CHIT1 was weak/absent in normal cartilage, while the expression for both CHI3L1 and CHIT1 was very strong in osteoarthritic cartilage with ACLT. The two chitinase are typically produced in the lysosomes and subsequently secreted. Their production is closely related to an inflammatory process, and pro-inflammatory cytokines.<sup>61</sup> CHIT1 and CHI3L1 present Carbohydrate-binding motif (CBM), then the ability to bind carbohydrates, in particular glycosaminoglycans. This ability could explain the altered levels of these two molecules dur-

ing a chronic inflammatory process such as OA. This observation is consistent with the evidence showing that CHI3L1 inhibition restrains tumour growth and metastasis by its own CBM.<sup>62</sup> Furthermore, treatment with both CHIT1 and CHI3L1 siRNAs in osteoclast *in vitro* model, induced a significant reduction of MMP9.<sup>54,20</sup> Recent articles confirm that the gelatinases influence OA onset and progression regulating the subchondral bone remodeling. In particular, a predominant role of MMP-9 emerged during last year. Among various MMPs, the total MMP-9 level is positively correlated with the total MMP-13 level in OA,<sup>63,64</sup> and it has been hypothesized that this gelatinase might be involved in the activation of pro-MMP-13 through yet unknown mechanisms. Notably, MMP-13 has long been considered as the major enzyme involved in cartilage erosion during OA, thus MMP-9 might play a role, at least cooperatively, in joint degradation. That being so, it is reasonable that the chitinase may be involved in the resorption of articular cartilage in OA through different pathways. The CHIT1 could be involved in the resorption of cartilage and bone matrix *via* its catalytic site and the CBM, while the CHI3L1 could activate pro-inflammatory cytokines through its CBM. The present finding indicates that CHI3L1 and CHIT1 could play an important role

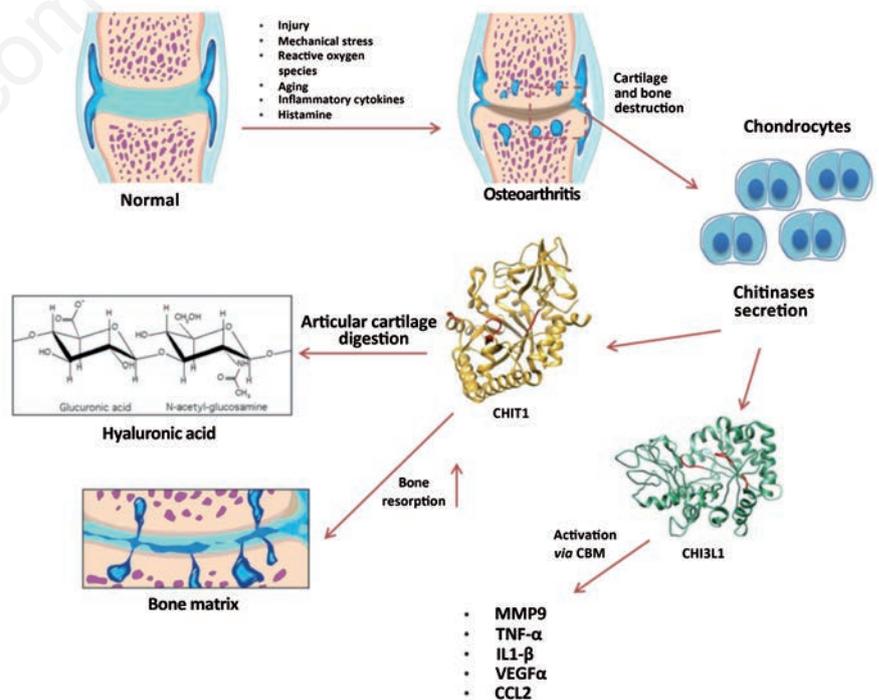


Figure 6. Graphic representation of CHI3L1 and CHIT1 expression in osteoarthritis.

in cartilage remodeling/degradation of osteoarthritic joints. CHIT1 and CHI3L1 may emerge as useful markers for OA and tissue degeneration. The expression of CHIT1 and of CHI3L1 in chondrocytes of osteoarthritic cartilage has a detrimental role in the cellular remodelling during the OA. These results are preliminary and further, longer-term *in vivo* and *in vitro* studies are needed to understand the exact mechanism of CHI3L1 and CHIT1 production and regulation in cartilage tissue and to define in detail these molecules, their receptors and activities on cells involved in OA. Serum and synovial fluid chitinases levels could be a marker of inflammatory cartilage diseases and since they correlate with the degree of joint destruction they could be used in the daily clinical practice to stage the severity of the disease.

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